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(21) International Application Number: PCT/US88/02483 (22) International Filing Date: 20 July 1988 (20.07.88) (31) Priority Application Number: 075,662 (32) Priority Date: 20 July 1987 (20.07.87) (33) Priority Country: US (60) Parent Application or Grant (63) Related by Continuation US 075,662 (CIP) Filed on 20 July 1987 (20.07.87) (71) Applicant (for all designated States except US): MARI-CULTURA, INCORPORATED [US/US]; Post Office Drawer 565, Wrightsville Beach, NC 28480 (US).		(72) Inventor; and (75) Inventor/Applicant (for US only) : LONG, Thomas, Veach, II [US/US]; 315-B Summer Rest Road, Wilmington, NC 28403 (US). (74) Agent: BARBER, Lynn, E.; Olive & Olive, P.O. Box 2049, Durham, NC 27702 (US). (81) Designated States: AT (European patent), AU, BB, BE (European patent), BG, BJ (OAPI patent), BR, CF (OAPI patent), CG (OAPI patent), CH (European patent), CM (OAPI patent), DE (European patent), DK, FI, FR (European patent), GA (OAPI patent), GB (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL (European patent), NO, RO, SD, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent), US. Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: MICROORGANISM PRODUCTION OF OMEGA-3 (N-3) LIPIDS (57) Abstract <p>The invention comprises the use of obligately and facultatively marine eukaryotic microorganisms for the production of Omega-3 (n-3) fatty acids that may be used in food, cosmetic, and pharmaceutical products. In the invention the microorganisms are grown heterotrophically, harvested, and extracted for lipid products.</p>		

MICROORGANISM PRODUCTION OF OMEGA-3 (N-3) LIPIDS

Field of the Invention

5 This invention relates to fatty acid production from microorganisms. In particular, this invention relates to the use of obligately or facultatively marine eukaryotic microorganisms grown heterotrophically and the production of a group of highly polyunsaturated fatty acids known as Omega-3 or n-3 fatty acids.

10 Background Information

The biosynthesis of fats and oils by microbes such as yeasts, bacteria, molds, and algae is well established, and inventors have devised ways of optimizing the growth conditions for this biosynthesis. For example, yeasts or
15 molds in mixed culture with bacteria and grown on mixtures of carbohydrates or hydrocarbons under aerobic fermentation conditions were found to produce numerous amino acids plus, in some cases, unspecified oil and fat. (U.S. Pat. No. 3,793,153). The disclosure of this reference and all others
20 cited herein are hereby incorporated by reference. Yeasts such as Candida and Rhodotorula also produce single-cell protein and undifferentiated lipid from vegetable carbohydrates including starch (U.S. Pat. No. 4,230,806). Under aerobic conditions Candida tropicalis produces
25 unsaturated dicarboxylic acids having 14 to 22 carbon atoms from unsaturated fatty acids or their esters (U.S. Pat. No. 4,474,882). Numerous yeasts dramatically increase their production of 1, 3-disaturated-2-unsaturated triglycerides, a predominant component of cacao butter, under high oxygen
30 fermentation conditions in a growth medium containing one or more fatty acids having between 10 and 20 carbon atoms. (U.S. Pat. No. 4,485,173). Rhodococcus rhodochrous (formerly

appear to be an essential nutrient. Although an earlier fermentation process of the prokaryotic *Streptomyces* organisms (U.S. Pat. No. 3,127,315) produced a hypocholesterolemic agent referred to as M-850, it is clear from the extraction procedures used that this agent is not related to the Omega-3 fatty acids or their action.

The focus of medical research on the fatty acids has been principally on two of the Omega-3 fatty acids, eicosapentaenoic acid (EPA: a 20-carbon fatty acid having 5 unsaturations) and docosahexaenoic acid (DHA: a 22-carbon fatty acid having 6 unsaturations), although others of this class may prove to be important. It is widely recognized that the principal dietary sources of these chemical moieties for fish are photosynthetic algae and microalgae. The use of the marine microalga, Chlorella minutissima, to produce Omega-3 fatty acids photoautotrophically has been the subject of recent patents (U.S. Pat. No. 4,615,839 and Jap. Pat. Discl. No. Sho-61-63624). A process for preparation of eicosapentaenoic acid from linolenic acid using enzymes from microalgae and macroalgae also has been described (Jap. Pat. Discl. No. Sho-61-31092).

In contrast, no method has been described heretofore to produce Omega-3 fatty acids from microbes that are grown heterotrophically, using as a nutrient a sugar, carbohydrate, or other source of "pre-formed" carbon. Although a process for cultivating the freshwater microalga Chlorella mixotrophically on lower fatty acids to aid in disposing of organic wastes (U.S. Pat. No. 3,444,647) was developed, no product description is provided, and this species does not produce Omega-3 fatty acids.

Summary of Invention

This invention comprises use of certain heterotrophic and autotrophic eukaryotes grown heterotrophically to produce

or one containing natural or artificial seawater, will produce Omega-3 fatty acids, including but not limited to EPA and DHA, as a significant percentage of total lipid. Furthermore, the use of such microorganisms yields a lipid fraction that is useful as a nutritional supplement; as a food additive in margarines, cooking oils, salad dressings, baked products, infant nutritional formulae and adult enteral nutritional formulae; as a skin care product or cosmetic; as a drug or pharmaceutical; as a component of an intravenous, parenterally administered fluid; and as an animal or aquaculture feed or feed additive. The high levels of Omega-3 fatty acids produced by these marine eukaryotes grown heterotrophically are unique. Prokaryotic microorganisms generally do not produce these fatty acids. Although photosynthetic, autotrophic eukaryotes do synthesize the Omega-3 fatty acids, the rates of growth of these organisms and their production of the fatty acids under photosynthetic growth conditions are significantly less than when heterotrophic eukaryotes are cultivated heterotrophically. Specific heterotrophs appropriate for use in this invention include, but are not limited to: the thraustochytrids, Thraustochytrium roseum and T. aureum; the phycomycetes fungi, Pythium sp. and Schizochytrium aggregatum; the diatom, Nitzschia sp.; and the dinoflagellate, Crypthecodinium cohnii. These species are maintained in the American Type Culture Collection and the algae culture collection of the University of Texas at Austin.

The Omega-3 fatty acids appear to be produced only by marine microorganisms or by halophilic or halo-tolerant species. Thus, in the preferred embodiment, either seawater, an artificial seawater, or other saline solution is used as the solvent in the culture medium. The complete medium is referred to as a saline culture medium. The carbon source in this saline culture medium may be a relatively simple carbohydrate source, for example, glucose, sucrose, mannose, or molasses, or, if slower cultivation is permitted, vegetable fibers such as grasses or bagasse.

Example 2

The procedures followed in Example 1 are used, except that the saline culture medium is composed of 1 to 5 g glucose, 1 g yeast extract, and 1 g peptone in 1 liter of seawater. The pH is adjusted to between 7 and 7.5.

Example 3

The procedures followed in Example 1 are utilized, except that an artificial seawater base is prepared for this saline culture medium. This consists of 2.5 g NaCl, 0.5 g MgSO₄·7H₂O, 0.1 g KCl, 0.01 g KH₂PO₄, 0.02 g CaCO₃ and sufficient H₂SO₄ to dissolve the above compounds. To this solution, (NH₄)₂SO₄ is added in the amount of 0.02 g, along with 0.2 g NaH-glutamate, 0.1 g Agar, 1.0 ug Thiamine-HCl, 0.1 ug Cyanocobalamin, 5.0 mg Na₂EDTA, and the trace metals 0.05 mg FeSO₄·7H₂O, 0.02 mg ZnSO₄·7H₂O, 0.01 mg MnSO₄·H₂O, 2.0 ug CoSO₄·7H₂O, 0.2 ug CuSO₄·5H₂O, 2.0 ug H₃BO₃, 2.0 ug NaMoO₄·H₂O with sufficient distilled water to yield 100 ml of solution. To this is added 0.1 to 0.5 g glucose or other sugar, 0.01 g NaHCO₃, and the pH is adjusted to between 7 and 7.5.

Example 4

The media and procedures employed in any of the above examples are used except that the cultures are exposed to light of moderate intensity. Heterotrophic growth of certain marine eukaryotes, such as the thraustochytrids, is enhanced under such conditions.

Example 5

The media and procedures employed in any of the above examples are used except that additional limited quantities of available N and P (such as 0.085 g NaNO₃ and 0.012 g NaH₂PO₄) are added to the saline culture medium. Heterotrophic growth

What is claimed is:

1. A process of using heterotrophically grown obligately or facultatively marine eukaryotic microorganisms as a source for the production of Omega-3 (n-3) fatty acids.
- 5 2. A process according to claim 1, wherein the marine eukaryotic microorganism is selected from the group consisting of thraustochytrids, lower fungi, yeasts, and microalgae.
- 10 3. A process for the production of Omega-3 (n-3) fatty acid products from obligately or facultatively marine eukaryotes, comprising the steps of:
 - (a) inoculating a saline culture medium containing a carbon source with marine eukaryotic microorganisms;
 - 15 (b) incubating the inoculated saline culture medium under conditions conducive to heterotrophic growth of the marine eukaryotic microorganisms;
 - (c) harvesting the marine eukaryotic microorganisms from the saline culture medium; and
 - 20 (d) extracting the lipid fraction from the harvested marine eukaryotic microorganisms.
4. A process according the claim 3, wherein the saline culture medium comprises seawater.
5. A process according to claim 4, wherein the carbon source comprises glucose.
- 25 6. A process according to claim 4, wherein the conditions conducive to heterotrophic growth comprise a pH of from 7

WO 89/00606

[received by the International Bureau on 12 January 1989 (12.01.89);
new claims 16-19 added; claims 1-15 unchanged (3 pages)]

16. A process for the production of Omega-3 (n-3) fatty acid products from obligately or facultatively marine eukaryotes, comprising the steps of:

- (a) inoculating a saline culture medium containing a carbon source with marine eukaryotic microorganisms;
- (b) incubating the inoculated saline culture medium under conditions conducive to heterotrophic growth of the marine eukaryotic microorganisms;
- (c) harvesting the marine eukaryotic microorganisms from the saline culture medium;
- (d) extracting the lipid fraction from the harvested marine eukaryotic microorganisms; and
- (e) using the lipid fraction to produce a nutritional additive to human diets.

17. A process for the production of Omega-3 (n-3) fatty acid products from obligately or facultatively marine eukaryotes, comprising the steps of:

- (a) inoculating a saline culture medium containing a carbon source with marine eukaryotic microorganisms;
- (b) incubating the inoculated saline culture medium under conditions conducive to heterotrophic growth of the marine eukaryotic microorganisms;
- (c) harvesting the marine eukaryotic microorganisms from the saline culture medium;
- (d) extracting the lipid fraction from the harvested

of the marine eukaryotic microorganisms;

- (c) harvesting the marine eukaryotic microorganisms from the saline culture medium;
- (d) extracting the lipid fraction from the harvested marine eukaryotic microorganisms; and
- (e) using the lipid fraction to produce an animal feed additive product.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X Y	Bulletin of the Japanese Society of Scientific Fisheries (Tokyo, Japan), Volume 46, issued 1980, (WATANABE ET AL.) "Relationship between Dietary Value of Brine Shrimp <u>Artemia salina</u> and their content of omega-3. Highly unsaturated Fatty acids", pages 35-41. See entire document.	15 12-13
Y	US, A, 4,661,343 (ZABOTTO) 28 April 1987, See columns 4 and 5 in particular.	14
X Y	Journal of Protozoology (Athens, Georgia), Volume 17, issued 1970 (HARRINGTON ET AL.) "The Polyunsaturated Fatty Acids of Marine Dinoflagellates", pages 213-219. See entire document.	1-3, and 5-8 4-6, 9 and 10
X Y	Comparative Biochemistry and Physiology, (Elmsford, New York), Volume 29, issued 1969, (ELLENBOGEN, B. ET AL.) "Polyunsaturated Fatty Acids of aquatic fungi: possible phylogenetic significance". pages 805-811. See entire document.	1-3, 7 and 10 4-6 and 8-9
X Y	Journal of Experimental Botany (Oxford, England) Volume 25, issued August 1974 (OPUTE, F.I.), "Lipid and Fatty Acid Composition of Diatoms" pages 823-835. See entire document.	1-3, 7 and 10 4-6 and 8-9
Y	Mycologia (Bronx, New York), Volume 55, issued 1963 (GOLDSTEIN, S.) "Studies of a new species of Thraustochytrium that displays light stimulated growth", pages 799-811. See entire document.	1-10